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(Article begins on next page)



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Integrating thermal tolerance, water balance and morphology: an experimental study on dung beetles

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Abstract

The impacts of extreme and rising mean temperatures due to climate change can pose significant physiological challenges for insects. An integrated approach that focuses on mechanisms of body temperature regulation, water balance and morphology may help to unravel the functional traits underpinning thermoregulation strategies and the most relevant trade-offs between temperature and water balance regulation. Here, we focused on four species of tunneler dung beetles as important providers of ecosystem services. In this experimental research, we first quantified two traits related to desiccation resistance and tolerance via experimental tests, and subsequently defined two levels of resistance and tolerance (i.e. low and high) according to significant differences among species. Second, we identified morphological traits correlated with water balance strategies, and we found that desiccation resistance and tolerance increased with small relative size of spiracles and wings. High levels of desiccation tolerance were also correlated with small body mass. Third, by integrating thermal tolerance with functional traits based on desiccation resistance and desiccation tolerance, we found that the species with the highest survival rates under elevated temperatures (*Euoniticellus fulvus*) was characterized by low desiccation resistance and high desiccation tolerance. Our results suggest shared physiological and morphological responses to temperature and desiccation, with potential conflicts between the need to regulate heat and water balance. They also highlighted the sensitivity of a large species such as *Geotrupes stercorarius* to warm and arid conditions with potential implications for its geographic distribution and the provisioning of ecosystem services under a climate change scenario.

Keywords

Upper thermal tolerance, desiccation resistance, desiccation tolerance, Scarabaeoidea, body mass, spiracle, climate change

Abbreviations

CT_{max} = upper critical temperatures

CT₅₀ = temperature at which 50% individuals experienced total paralysis

CT₁₀₀ = temperature at which 100% individuals experienced total paralysis

DR= desiccation resistance

DT = desiccation tolerance

S_{TEMP} = survival rates related to increasing temperatures

1. Introduction

The impacts of extreme and rising mean temperatures due to climate change can pose significant physiological challenges for many species (Helmuth, 2009; Morley et al., 2019), with implications for biodiversity conservation (Gunderson & Stilmann, 2015) and the provisioning of ecosystem services (Runting et al., 2017). Climate change represents one of the main threats to insect decline along with habitat loss and degradation and the spread of invasive species (Sánchez-Bayo & Wyckhuys, 2019; Wagner, 2021; van Klink et al., 2020). Such a decline is devastating given the ecosystem services that insects provide, and the critical role they play in food webs (Numa et al., 2020).

Because most insects are ectotherms with poikilothermic strategies, their physiology, development, and behavior are intimately linked to temperature. Physiological strategies become especially important under extreme conditions of cold and warm temperatures where behavioral responses are

not sufficient to offset potential negative effects. Thermal tolerance is an important physiological trait that affects heat flux between insect bodies and the surrounding environment (Chown et al., 2003; Chown & Terblanche, 2007; Rozen-Rechels, 2019) by determining survival of both larvae and adults under extreme conditions. Even though heat loss due to evaporative cooling across the cuticle is an uncommon strategy in insects because they tend to contain little water due to their size, it can be an important response mechanism for an insect exposed to temperature near the maximum tolerable (Harrison, 2012). Evaporative cooling depends both on cuticular water loss and respiratory water loss by diffusion and convection from open spiracles (Lighton, 1994; Heinrich, 1980; Hadley et al. 1989), and it generally increases with larger body surfaces (Harrison, 2012). Moreover, physiological responses to increasing temperatures include higher metabolic rates that depend on water-losing processes such as an increase in oxygen demands (which leads to respiratory water loss) (Nicolson & Louw, 1982; Willmer, 1988; Lehmann et al., 2000; Woods & Smith, 2010) and disposal of nitrogenous waste (i.e. urine water loss). Water availability and climate conditions, especially environmental temperature, vary widely in space and time, influence the energy and water budgets of terrestrial insects, and affect functional adaptations to cope with temperature and water constraints (Angilletta, 2009; McKinley et al., 2018; Mole et al., 2016; Sears et al., 2016). Insects have a variety of mechanisms to increase desiccation resistance (i.e. ability to reduce water loss). First, larger insects tend to have lower surface area to volume ratio, more water storage, and more fat that can be converted to metabolic water (Hadley 1994). Second, insects can actively slow water loss by, for example, closing spiracles, or increasing rectal water reabsorption (Harrison et al. 2012). Third, composition, quantity, and physics of cuticular hydrocarbons that form a barrier of hydrophobic bonds may increase desiccation resistance (Edney, 1977; Jurenka et al., 2007; Bazinet et al., 2010). In addition to these mechanisms, insects can enhance desiccation tolerance (i.e. ability to tolerate water loss) by production of cellular compounds which maintain cell function under dehydration stress (e.g., heat shock proteins, Lopez-Martinez et al., 2009; Benoit & Denlinger, 2010). Thermoregulation and water balance closely interact (Rozen-Rechels et al., 2019), and may involve shared behavioral and physiological responses influenced by functional trade-offs between the need to regulate body temperature and water balance at the same time. An integrated approach that considers temperature and water balance together is needed to avoid limited insights into insect responses to climate change (e.g., Bonebrake & Mastrandrea, 2010; Clusella-Trullas et al., 2011).

In common with other insects (Angilletta et al. 2004; Chown et al. 2004), dung beetles (Coleoptera: Scarabaeoidea), which feed on mammalian dung and breed in dung or soil based on their reproductive strategy, are strongly affected by temperature. Differences in oviposition and nesting behaviour (i.e. tunnelers, rollers and dwellers, Hanski & Cambefort, 1991) result in species that may experience different environments and respond differently to climate changes. Indeed, tunnelers and rollers dig tunnels in soil for nesting and breeding, while dwellers live and breed in dung (Slade & Roslin, 2016). Direct sunlight can rapidly heat-up small areas of dung/soil to temperatures that exceed the heat tolerance of beetles, which creates heterogeneity in soil thermal conditions that influence the beetles' local spatial distribution, habitat use, daily and seasonal activity patterns (Gómez et al. 2020, Cuesta et al., 2021), reproduction (Holley & Andrew 2019, Holley & Andrew, 2020), distribution and community composition (Lobo & Martín-Piera 2002a; Dortel et al., 2013; Cuesta & Lobo 2019, Nyamukondiwa et al. 2018).

While interactions between thermoregulation and water balance in dung beetles have been poorly explored, several studies have focused on physiological mechanisms of heat production and loss. Previous studies have shown that some dung beetle species [e.g. *Geotrupes stercorarius* - Krogh & Zeuthen (1941)] have the ability to produce endogenous heat by using their wing muscles prior to take-off, during flight, and during ball making and ball rolling, that increases body temperature, particularly of the thorax (Bartholomew & Heinrich, 1978; Verdú et al., 2004; Verdú et al., 2006; Verdú et al., 2007; Verdú & Lobo, 2008; Gallego et al., 2018). Temperature deviations between body regions, termed regional heterothermy, have been observed in many insects (Chown et al., 1995; Woods et al., 2005). Heinrich & Bartholomew (1979) suggested a relationship between the ability to thermoregulate and body size, with larger beetles more effective in increasing their body temperature (e.g. Verdú et al., 2006). This mechanism may influence thermal niche partitioning and geographical distribution patterns (Verdú & Lobo, 2008). However, in order to regulate body temperature, insects not only have to increase their body temperature, but also must sometimes decrease it in order to stay within the limits of physiological activity. Different dung beetle species may have different thermoregulatory mechanisms to transfer heat from thorax to abdomen to avoid overheating (e.g. abdominal heat transfer) or cooling (e.g. thorax heat retention) (Verdú et al., 2012).

In a context of increasing intensity and frequency of extreme thermal events (Sheridan & Allen, 2015), understanding how thermal tolerance and water balance of dung beetles control their

distribution and community composition, and consequently their effects on ecosystem services, is especially important. Here, we used four species of tunneler dung beetles (i.e. they dig tunnels underneath dung pads and construct breeding chambers with dung provisions that are consumed by their larvae) as important ecosystem service providers (e.g. dung removal, nutrient cycling, parasite suppression; Nichols et al. 2008) to explore the interactions between temperature regulation, water balance, and morphology. First, we quantified two traits related to desiccation resistance and desiccation tolerance via experimental tests and, according to significant differences among species, we defined two levels of resistance and tolerance (i.e. low and high). Second, we identified morphological traits correlated with water balance strategies. Third, we identified the upper thermal tolerance limit of the four dung beetle species by measuring upper critical temperatures (CT_{max}, the temperature causing total paralysis) via thermal test experiments in a Peltier-cooled incubator, and related thermal tolerance to previously defined traits based on desiccation resistance and desiccation tolerance. We expected that, if increased thermal tolerance and water balance regulation require different costly investments, then this could result in a trade-off between one investment over the other. For example, evaporative cooling enables insects to tolerate higher temperatures, but results in high water loss (Renault et al. 2005); therefore, individuals with high tolerance to water loss may have an advantage in extremely warm conditions. In conclusion, we discuss the potential implications of the interactions among thermal tolerance, water balance and morphological traits in the framework of dung beetle conservation and functional ecology.

2. Materials and Methods

2.1. Species collection and trait measurements

Individuals of four dung beetle species belonging to the Geotrupidae and Scarabaeidae families (*Geotrupes stercorarius*, *Onthophagus fracticornis*, *O. taurus* and *Euoniticellus fulvus*) were collected in September 2019 in three areas of Piedmont (north-west Italy) from cattle and sheep dung (Table 1). These species are able to utilize a wide range of dung from herbivorous mammals, especially cattle, horse and sheep dung (Dormont et al., 2006). 87 individuals were used for desiccation resistance and tolerance measurements, and 50 individuals were used for measurements of thermal tolerance.

2.1. Desiccation resistance and desiccation tolerance measurements

Desiccation resistance and tolerance were measured by following a trait-based protocol for measurement of traits (Moretti et al., 2017). We measured desiccation resistance and tolerance on 14-29 individuals per species (Table 1). Before measurements, individuals were kept in colonies of the same species inside plastic buckets filled with a mixture of humus for gardening and sand, and they were provided with *ad libitum* food (i.e. cattle dung) and water for a week to minimize effects of prior environmental conditions. After this period, before exposing the beetles to dry conditions for desiccation measurements, a pre-treatment procedure to replenish any possible water deficit was carried out without food in order to control for water loss from excretion. Beetles were kept isolated in small cylinders (diameter 2 cm, height 3 cm) placed in a closed glass box (40 x 20 x 20 cm), on top of a 3 cm layer of moist floral foam for 3 days, ensuring constant conditions of 100% relative humidity (RH). The laboratory was kept at a temperature of 20 °C for the whole acclimation period (average temperature: 20.4±0.3 °C) under a 12:12 h photocycle. After this procedure, insects were exposed to realistic dry conditions, approximately 70% RH (average value: 69.4±1.3%), to record survival time (an estimate of desiccation resistance) and percentage of fatal water loss rate (an estimate of desiccation tolerance). This RH value was chosen to represent a moderate stress condition considering that, on average, cattle dung has a relative humidity of 80% (Holter et al., 2016), and that 91-93% is the threshold above which arthropods (e.g. terrestrial isopods, beetles) are able to absorb water vapour (Wright & Machin, 1990; Hansen et al., 2006). The humidity level of 70% RH was reached using a glycerol–water solution in volume concentration of 48% (i.e., Dias et al., 2013). Plastic glasses (200 ml) were filled with 80 ml of glycerol solution. In each glass, a platform made of metal wire was placed about 1 cm above the solution's surface; the cylinder containing the animal was then placed on this platform and the glass closed with a plastic cover. The cylinders were open at both sides and covered with a nylon mesh cloth (width 0.5 mm) to prevent beetles from escaping, but allowing an adequate airflow between the solution and the cylinder inside the glass. The temperature range for the experimental period was 21-22 °C.

Before exposing the beetles to dry conditions, individuals inside the cylinders were weighed to record the initial fresh mass using an analytical balance (Precisa 125 A, 0.1 mg). We checked if dung beetles were alive at four time points per day (9:00 am, 12:00 am, 3:00 pm, 6:00 pm) by disturbing them gently with a soft brush or by flipping them with tweezers. The test was conducted for 96 hours, after which we ended the experiment, even if some individuals were

still alive, to avoid other potential stress factors such as starvation influencing the measurements.

Desiccation resistance was estimated as survival time, which refers to the time that dry conditions can be tolerated before an organism dies. Survival time of each individual was calculated as the number of hours an organism survives in proportion to the total number of hours of the experiment, expressed as a value between 0 (0h/96h) and 1 (96h/96h). If individuals died overnight, the median of the values (in hours) of the last measurement in the afternoon (e.g. 6:00 pm) and the first in the morning (e.g. 9:00 am) was used to calculate desiccation resistance, according to Dias et al. (2013). Desiccation resistance was measured in all of the individuals investigated. Desiccation tolerance was measured as a percentage of fatal water loss, and it was expressed as the proportion of the initial water content that was lost at the time of death, i.e. $[(\text{initial wet body mass} - \text{final wet body mass}) / \text{initial water content}] \times 100$. Initial water content was calculated as the initial wet body mass – dry body mass. If the individual died overnight (approximately 30% of individuals), we used the average value of body mass of the last measurement in the afternoon and the first in the morning. Desiccation tolerance was measured only in individuals that died during the experiment (Table 1). Furthermore, fractional water content (initial water content / dry body mass) was calculated to ensure that differences in desiccation resistance and tolerance among species were not linked to differences in initial water content.

2.2. Morphological measurement

From the sample of 87 individuals tested for desiccation resistance and tolerance (i.e. 14 individuals of *E. fulvus*, 27 of *G. stercorarius*, 29 of *O. fracticornis*, 17 of *O. taurus*), we measured dry body mass, abdominal spiracle size (ASS), mesothoracic spiracle size (MSS), and wing size (WS) of each individual.

To measure the dry body mass (hereafter *body mass*), the insects were dried in the oven (Binder FD) at 25°C for 24 hours and weighed using an analytical balance (Precisa 125 A, 0.1 mg) until the weight was constant between subsequent measurements (every 12 hours).

We measured the diameter of the six abdominal spiracles, the surface area of the mesothoracic spiracle, and the wing length. Since abdominal spiracles are small and of circular shape, the diameter of the six spiracles (expressed in mm) was considered as a proxy for their surface area.

The mesothoracic spiracle is kidney-shaped, thus the diameter could not be calculated, and the surface area was instead measured (expressed in mm²). ASS and MSS were expressed in relation to the pronotum width of each individual to take into account differences in size among species (Figure 1). The maximum pronotum width (expressed in mm) was considered as an acceptable proxy for body size. The right hindwing was measured from the articulation point to the apical hinge (i.e., subcostal vein) (Figure 1). The wing subcostal length was considered a good proxy for the surface area of the folded wings below the elytra.

To measure ASS, MSS, and WS, the abdomen tergites and the hindwings were dissected following the methods usually employed in dung beetles (Roggero et al., 2015), then the anatomical parts were mounted on slides, and photos of the structures were taken. The 2D images of the specimens and anatomical parts were captured by the software LAS-Leica Application Suite (Leica Microsystems AG, Wetzlar, Germany), using a Leica® DMC4500 (Leica Microsystems AG, Wetzlar, Germany) digital camera connected to a stereoscopic dissecting scope Leica® Z16APO (Leica Microsystems AG, Wetzlar, Germany). All the measurements were done by the LAS Measurement Module of the software Leica Application Suite (LAS). Average values of body mass, ASS, MSS, WS can be found in Table A1 of the Supplementary Materials.

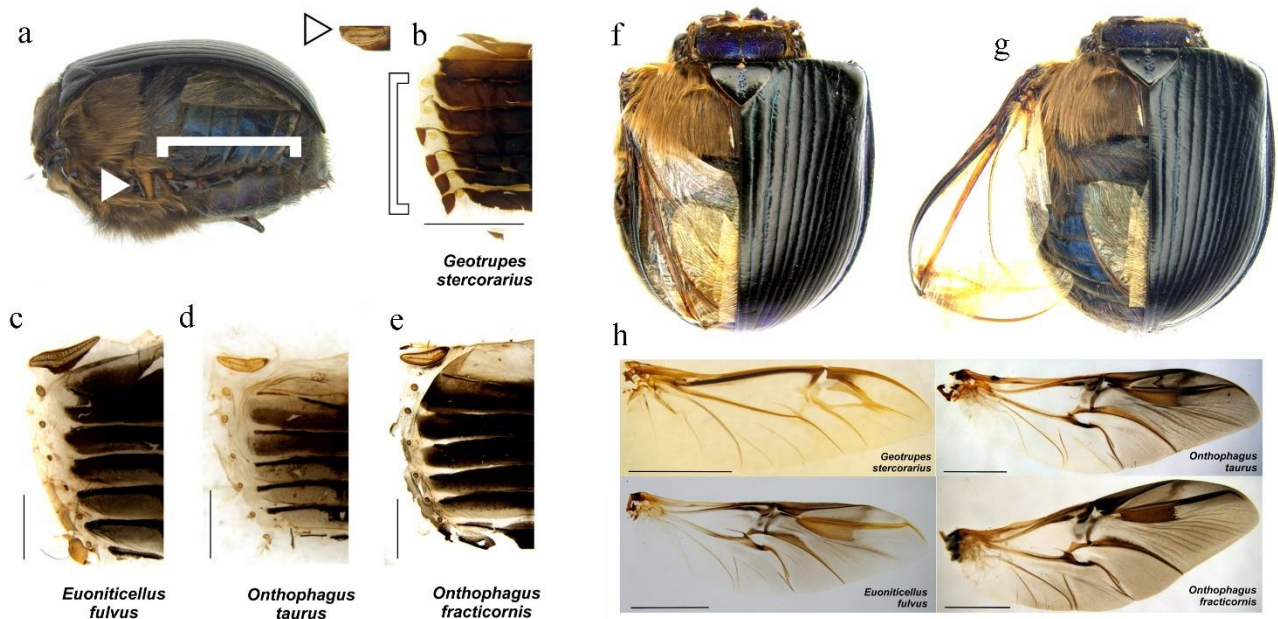
Potential effects of collinearity among morphological predictor variables (i.e. body mass and relative size of ASS, MSS and WS) were calculated using the pairplot combined with correlation coefficients in R (Zuur et al., 2009). Body mass showed high levels of collinearity with ASS, MSS, and WS, and ASS showed high levels of collinearity with MSS and WS (Figure A1 in the Supplementary Materials). We ran therefore separate models for body mass and ASS (see Statistical Analysis).

2.3. Thermal tolerance measurements

We measured thermal tolerance on 8-19 individuals per species depending on the abundance of the animals collected in the field (Table 1). Before measurements, dung beetles were acclimated for one week with *ad libitum* food and water at the same conditions of the acclimation procedure described above. In the laboratory, we placed individuals in a Memmert IPP 30 Peltier-cooled incubator, inside individual Petri dishes covered with a lid. Air humidity was kept at 100% during the experiment by placing two Petri dishes filled with water inside the incubator. We conducted an experiment to determine the upper critical temperature (CT_{max}), starting from 30°C, which is the

mean of maximum temperatures in summer 2019 in Piedmont (the fourth hottest season in the last 62 years – data from Regional Agency for Environmental Protection, Arpa Piemonte). In this regard, we considered 30°C as an appropriate starting point for the experiment, since we used individuals acclimated to the summer season. Temperature was gradually increased at a constant rate of 1°C/30 minutes. We evaluated the individual CT_{max}, that is the temperature at which the individual has lost the ability to move any body part, i.e. total paralysis (Le Lann et al., 2011). We observed that after the heating process, dung beetles never recovered after being removed from the incubator, a typical pattern when the temperature at which heat stupor occurs is close to the lethal temperature (Wu & Wright, 2015). We expressed the CT₅₀ as the measure of temperature at which 50% of individuals experienced total paralysis. The experiment continued until all the individuals had stopped moving (CT₁₀₀). For each species tested, we kept approximately 10% of the individuals as controls in stable climatic conditions, to exclude the confounding effect of causes of mortality other than temperature alteration (e.g., stress). No mortality was recorded in controls during the experiments. The experiments lasted 9.5 hours, and the conditions of individuals were checked every half an hour (19 checks). Individuals were not fed during the experiments. Survival rate of each individual related to increasing temperature (S_{TEMP}) was calculated as the number of checks an individual survived over the total number of checks, expressed as a value between 0 (0/19) and 1 (19/19).

Figure 1. Morphology of spiracles and hindwings: (a) position of abdominal and mesothoracic spiracles in *Geotrupes stercorarius* (side view); (b-e) abdomen with the spiracles (dorsal view) of *G. stercorarius* (scalebar =5.0 mm), *Euoniticellus fulvus*, *Onthophagus taurus* and *O. fracticornis* (scalebars = 1.0 mm). (f-g) *G. stercorarius* (dorsal view) abdomen with the left hindwing folded (f) and partially unfolded (g). (h) Right hindwing of *G. stercorarius* (scalebar = 5.0mm), *E. fulvus*, *O. taurus* and *O. fracticornis* (scalebars = 2.0mm).



2.4. Statistical analyses

All statistical analyses were conducted using R software (R Core Team, 2017). We tested for differences in desiccation resistance and tolerance between species (i.e. species was the predictor variable) by using Generalised Linear Models (GLMs) with a Binomial distribution for desiccation resistance (DR) and a Gaussian distribution for desiccation tolerance (DT). Shapiro–Wilk normality tests and Q–Q plots confirmed normality in the residual distribution for DT. Pairwise differences between the species were calculated using the ls means package in R (Lenth, 2016). According to the significant differences of desiccation resistance and tolerance among species, we defined for each species two functional traits based on DR and DT. These functional traits were based on two levels: low or high desiccation resistance and low or high desiccation tolerance (see Table 1). Differences among species in the fractional water content were also tested using a GLM with a Gamma distribution.

The extent to which overall differences in desiccation resistance and tolerance between species were driven by specific morphological traits was then assessed by replacing species with body mass, ASS, MSS, and WS as continuous predictors in the same models. Akaike’s Information Criteria (AICs) of species models and trait models were compared to assess the best fitting models. Body mass and ASS were tested in separate models from mesothoracic spiracle and wing

size because of their high levels of collinearity. Models with the lowest AIC were considered as the best fitting models (those within 2 of the top ranked model). Explained deviance was also considered as a goodness of fit measure: $1 - (\text{residual deviance} / \text{null deviance})$. Morphological differences among the four species (i.e. predictor variable) were also tested with GLMs. Shapiro–Wilk normality tests and Q–Q plots confirmed normality in the residual distribution for MSS and WS. A Gamma error distribution was specified in ASS and body mass, as continuous but non-normal dependent variables (Zuur et al., 2007).

We constructed standard survival curves in response to increasing temperature with binomial generalized linear models (GLMs), whereby we modelled the survival rate of each species (S_{TEMP}) as a function of temperature (i.e. as a continuous predictor variable). To test the differences in the upper thermal tolerance among the four species (i.e. as a four-level categorical predictor variable), we used GLMs with a Gamma distribution.

Table 1. Functional classification of the species collected according to their thermal tolerance and their desiccation resistance and tolerance. N(CT) is the number of individuals collected and tested for thermal tolerance, N(DR/DT) is the number of individuals collected and tested for desiccation resistance and tolerance. The numbers in parenthesis indicate the number of dead individuals during the experiment. $CT_{50/100}$ = temperature at which 50% or 100% individuals experienced total paralysis. Species were classified in functional groups according to their desiccation resistance (DR) and tolerance (DT) levels (low, high).

FAMILY	SPECIES	N (CT)	CT ₅₀	CT ₁₀₀	N (DR/DT)	Functional trait (DR)	Functional trait (DT)
Geotrupidae	<i>Geotrupes stercorarius</i> (Linnaeus, 1758)	8	40°C	41°C	27 (22)	Low	Low
Scarabaeidae	<i>Onthophagus fracticornis</i> (Preyssler, 1790)	10	40°C	45°C	29 (9)	High	High
	<i>Onthophagus taurus</i> (Schreber, 1759)	13	43°C	45°C	17 (8)	High	High
	<i>Euoniticellus fulvus</i> (Goeze, 1777)	19	47°C	48°C	14 (8)	Low	High

3. Results

3.1. Experimental test of desiccation resistance and tolerance

Desiccation resistance was higher in *O. fracticornis* and *O. taurus* compared to *E. fulvus* and *G. stercorarius*. Desiccation tolerance was higher in *O. fracticornis*, *O. taurus* and *E. fulvus* compared to *G. stercorarius* (Table 2, Figure 2). Based on these results, we defined for each species two functional traits (i.e. DR and DT) based on high (i.e. *O. fracticornis*, *O. taurus*) and low levels (i.e. *E. fulvus*, *G. stercorarius*) of desiccation resistance, and high (i.e. *E. fulvus*, *O. fracticornis*, *O. taurus*) and low levels (*G. stercorarius*) of desiccation tolerance (Table 1). The four species showed similar fractional water content (Table A2 in the Supplementary Materials), suggesting that differences in desiccation resistance and tolerance among species were not linked to differences in initial water content.

Desiccation resistance was not influenced by body mass and MSS, while it was affected by ASS and WS (Table 3). Desiccation resistance increased in individuals with smaller abdominal spiracles and shorter wings relative to body size. The best fitting model for desiccation resistance included wing size. Desiccation tolerance was influenced by body mass, ASS, MSS, and WS (Table 3). Desiccation tolerance increased in small individuals with smaller spiracles and shorter wings relative to body size. The best fitting model for desiccation tolerance included abdominal spiracle size.

AIC comparisons between models including continuous predictors (i.e. body mass, ASS, MSS, and WS) and single species models (Table 2) showed that the best model for desiccation resistance included wing size instead of species identity ($\Delta\text{AIC}= 12$), indicating the differences between species are potentially driven by morphological traits such as size of wings and spiracles. Even though morphological traits significantly influenced desiccation tolerance, the best model for DT included species identity ($\Delta\text{AIC}= 6$), suggesting that additional unmeasured variables are also important in driving species-specific responses to desiccation tolerance.

Specifically, *G. stercorarius*, characterized by low levels of desiccation resistance and tolerance, was significantly heavier than the other three species, and showed larger relative size of abdominal spiracles, mesothoracic spiracles, and wings, while *E. fulvus*, *O. fracticornis* and *O. taurus* did not differ significantly in body mass. *O. fracticornis*, one of the most resistant and tolerant species to

water loss, showed the smallest size of relative abdominal and mesothoracic spiracles compared to the other three species. *O. taurus* showed shorter wings relative to body size than *E. fulvus* and *O. fracticornis* (Table A3 in the Supplementary Materials, Figure 2).

Table 2. Differences among species in desiccation resistance and tolerance. The reference level for species is *E. fulvus*.

Desiccation resistance (~ species)			
AIC= 683 (sample size = 76)			
Variance explained= 0.17			
	Estimate	t value	p
Intercept	-0.32±0.04	-7.5	***
<i>G. stercorarius</i>	0.01±0.05	0.11	NS
<i>O. fracticornis</i>	0.25±0.05	4.77	***
<i>O. taurus</i>	0.20±0.06	3.66	***
Species comparisons			
<i>G. stercorarius</i> - <i>O. fracticornis</i>	-0.24±0.04	-5.75	***
<i>G. stercorarius</i> - <i>O. taurus</i>	-0.19±0.05	-4.25	***
<i>O. fracticornis</i> - <i>O. taurus</i>	0.04±0.05	0.94	NS
Desiccation tolerance (~ species)			
AIC= 320 (sample size = 41)			
Variance explained= 0.56			
	Estimate	t value	p
Intercept	69.67±3.87	18.00	***
<i>G. stercorarius</i>	-19.28±4.47	-4.32	***
<i>O. fracticornis</i>	9.74±5.70	1.71	NS
<i>O. taurus</i>	-5.21±5.23	-0.98	NS
Species comparisons			
<i>G. stercorarius</i> - <i>O. fracticornis</i>	-29.02±4.74	-6.12	***

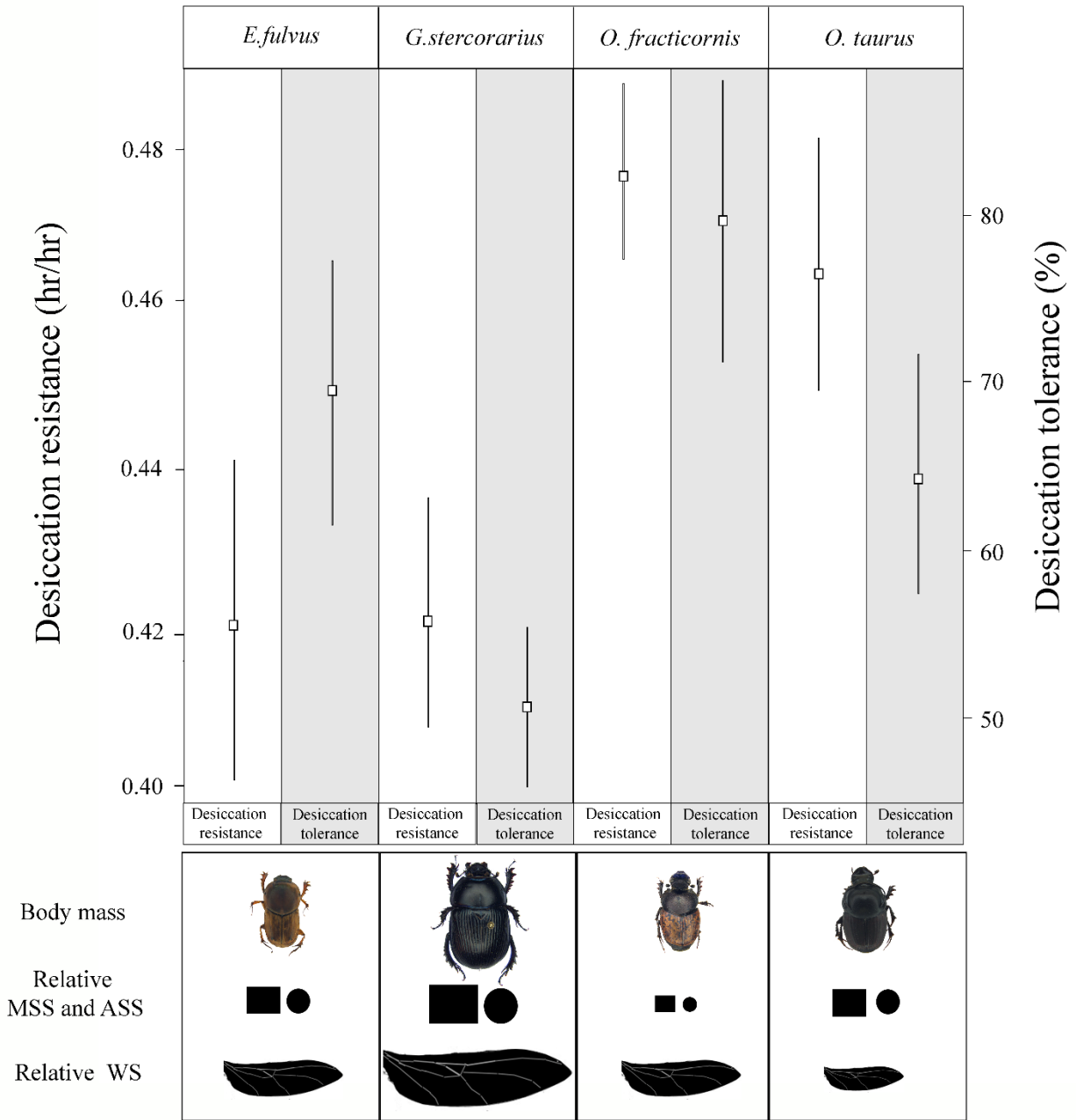
<i>G. stercorarius</i> - <i>O. taurus</i>	-14.08±4.25	-3.31	**
<i>O. fracticornis</i> - <i>O. taurus</i>	14.94±5.53	2.70	*

Table 3. Effects of morphological variables (body mass, and relative size of abdominal spiracles, mesothoracic spiracle, and wing) on desiccation resistance and tolerance.

DR ~ Body mass				DT ~ Body mass			
AIC= 724 (sample size = 79)				AIC= 337 (sample size = 41)			
Variance explained= 0.005				Variance explained= 0.28			
	Estimate	t value	p		Estimate	t value	p
Intercept	-0.18±0.02	-8.75	***	Intercept	67.84±2.71	25.03	***
Body mass	-0.14±0.11	-1.24	NS	Body mass	-54.55±13.77	-3.96	***
DR ~ ASS				DT ~ ASS			
AIC= 707 (sample size = 79)				AIC= 326 (sample size = 41)			
Variance explained= 0.07				Variance explained= 0.44			
	Estimate	t value	p		Estimate	t value	p
Intercept	0.15±0.08	1.80	0.07	Intercept	109.66±8.97	12.22	***
ASS	-2.16±0.50	-4.27	***	ASS	-287.27±0.02	-5.59	***
DR ~ WS				DT ~ MSS + WS			
AIC= 671 (sample size = 79)				AIC= 327 (sample size = 41)			
Variance explained= 0.20				Variance explained= 0.46			
	Estimate	t value	p		Estimate	t value	p
Intercept	1.21±0.19	6.32	***	Intercept	144.27±19.25	7.49	***
WS	-1.81±0.25	-7.35	***	MSS	-640.03±196.21	-3.26	**

				WS	-68.59±27.03	-2.54	*
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Figure 2. Differences in desiccation resistance (white panel) and desiccation tolerance (grey panel) between the four species. Morphological characterization of the four species is represented below the table, based on body mass, and relative sizes of abdominal spiracles (ASS, black circle), mesothoracic spiracle (MSS, black rectangle) and wing (WS).



3.2. Experimental test of upper thermal tolerance

All the species were successfully tested in the Peltier-cooled incubator and classified according to their upper thermal tolerance values (Table 1). Survival curves showed different shapes for the four species, indicating *E. fulvus* as the most tolerant species to warm temperatures, followed by *O. taurus* and *O. fracticornis*. *G. stercorarius* was the least tolerant species (see CT_{50} and CT_{100} in Table 1) (Figure 3). These results were confirmed by the significant differences in the upper thermal tolerance of the four species (Table 4).

Figure 3. Tests on thermal tolerance. Estimated survival rate (S_{TEMP}) as a function of temperature. Survival curves represent best fits to the data, according to generalized linear models (GLMs).

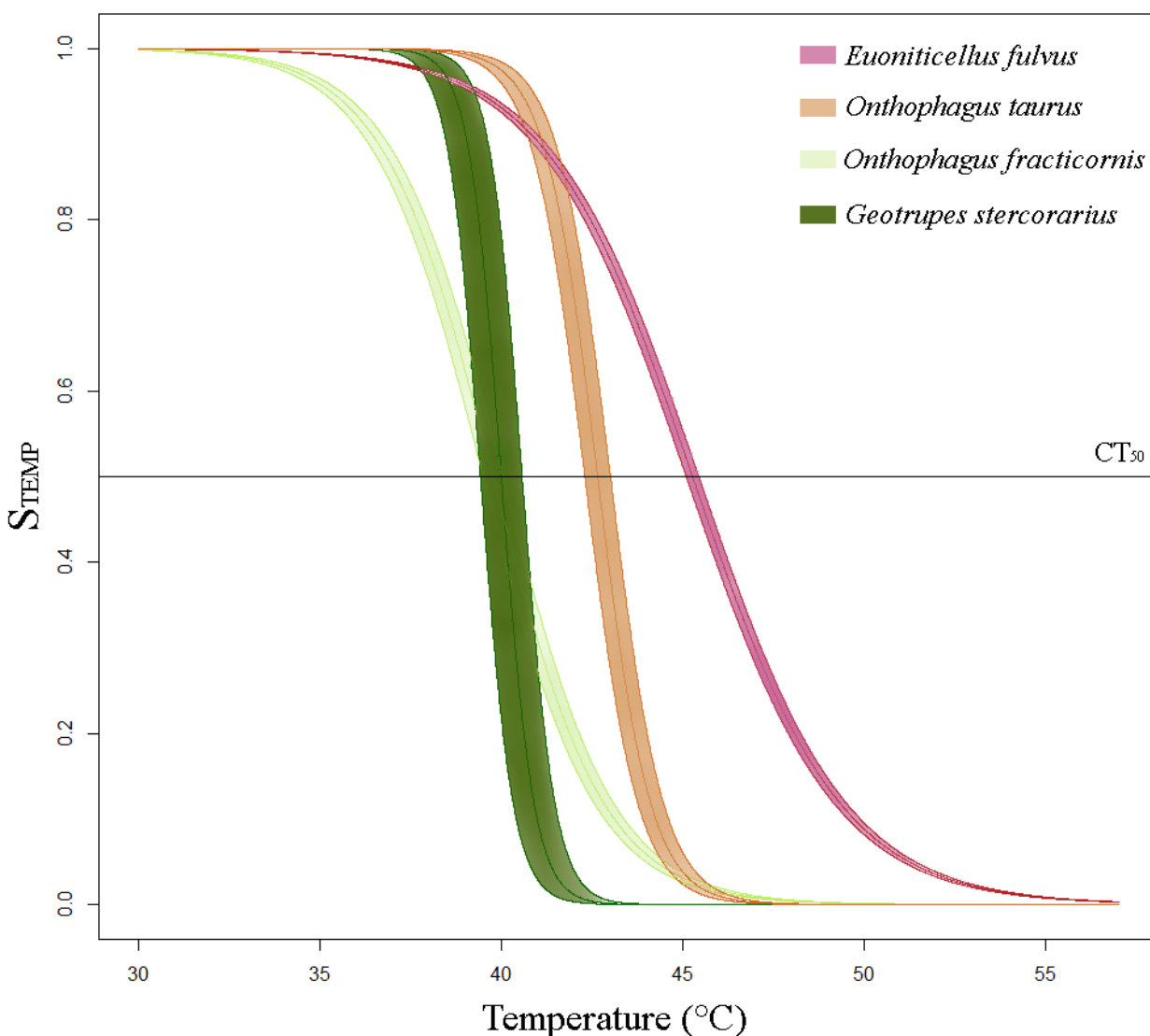


Table 4. Differences in the upper thermal tolerance (CT_{max}) among the four species. The reference level for species is *E. fulvus*.

CT _{max} ~ species			
Sample size = 49			
Variance explained= 0.82			
	Estimate	t value	p
Intercept	3.85±0.01	569.24	***
<i>G. stercorarius</i>	-0.15±0.01	-11.80	***
<i>O. fracticornis</i>	-0.13±0.01	-11.67	***
<i>O. taurus</i>	-0.08±0.01	-7.84	***
Species comparisons			
<i>G. stercorarius</i> – <i>O. fracticornis</i>	-0.12±0.01	-0.88	NS
<i>G. stercorarius</i> – <i>O. taurus</i>	-0.06±0.01	-4.79	***
<i>O. fracticornis</i> – <i>O. taurus</i>	-0.05±0.01	-4.13	**

4. Discussion

Understanding how insects respond to environmental challenges is critical in order to forecast the impacts of a globally changing climate on populations and communities. An integrated approach that focusses on mechanisms of body temperature regulation and water balance may help to unravel the functional traits underpinning thermoregulation strategies and the most relevant trade-offs between temperature and water balance regulation. This is particularly pressing for dung beetles which provide multiple ecological functions that are crucial to maintain viable ecosystems (Nichols et al., 2008; Beynon et al., 2012; Braga et al., 2013; Nervo et al., 2014; Braga et al., 2017; Nervo et al., 2017; Piccini et al., 2017a). Here, we first quantified traits related to desiccation resistance and tolerance via experimental tests and therefore defined categories of functional traits instead of inferring them from species distributions (see Cardillo, Dinnage, & McAlister, 2019). Second, we

identified morphological traits correlated with water balance strategies, and we found that desiccation resistance and tolerance increased with decreasing relative size of spiracles and wings. Moreover, smaller bodied beetles had higher desiccation tolerance. Third, by integrating thermal tolerance with physiological traits based on desiccation resistance and tolerance, we found that the species with the highest survival rates under elevated temperatures (*E. fulvus*) was characterized by low desiccation resistance and high desiccation tolerance. Our results provide support to our hypothesis that suggests shared physiological and morphological responses to temperature and water, with potential conflicts between the need to regulate heat and water balance.

4.1. Desiccation resistance and tolerance, and the underlying morphological traits

G. stercorarius, characterized by larger body mass and larger relative size of spiracles and wings compared to the other three species, showed lower resistance and tolerance to water loss. Previous studies have shown that larger body size reduces the surface area-to-volume ratio (Schmidt-Nielsen & Knut, 1984; Chown et al., 1998), thus improving water conservation. This may represent an advantage in terms of a reduced water loss rate (Hadley, 1994), since body surface area does not increase linearly with body mass or volume. However, our study suggested that the advantage of a large body size in favouring desiccation resistance and tolerance may be counterbalanced by larger spiracles and wings that increase water loss via transpiration. These results are supported by the evidence that no differences in fractional water content were found among the four species, suggesting that differences in desiccation resistance and tolerance were not determined by the reserves of water storage in the studied beetles. Related species such as *O. fracticornis* and *O. taurus* of the Scarabaeidae family had small spiracles and wings relative to their body size, and consequently high levels of resistance and tolerance to water loss. *E. fulvus*, which showed low resistance but high tolerance to water loss, shared similar body mass values with the two *Onthophagus* species, but had a larger relative size of spiracles compared to *O. fracticornis*, and a larger relative wing size compared to *O. taurus*. The Scarabaeidae family constitutes the main thermophilous group of European dung beetles (Lumaret et al., 1991; Lobo et al., 2002a; Lobo et al., 2002b); their ability to resist and tolerate arid conditions may be linked to their small spiracle and wing size compared to their body size.

It is interesting to note that a small amount of variance in desiccation resistance was explained by spiracle and wing size (see Table 3), even though continuous variables were better predictors than

species identity. For desiccation tolerance, single species models were better models compared to those including abdominal spiracles (see Table 2 and 3). This result suggests that traits other than those considered may be more important in providing resistance to dehydration in dry environments, for example cuticular hydrocarbons, which form a barrier of hydrophobic bonds that increase desiccation resistance (Edney, 1977; Jurenka et al., 2007; Bazinet et al., 2010).

In common with our findings, previous studies on other taxa have also identified spiracle size as one of the mechanisms conferring tolerance to water loss in insects (e.g. Anophelinae mosquitos in Arcaz et al., 2016). Spiracles are major respiratory openings in the exoskeleton that allow fluxes of oxygen (O₂) and carbon dioxide (CO₂) to and from muscles, and therefore they may represent potential weak points of respiratory water loss. The potential threat of desiccation is greatest during flight, when the spiracles are not closed by the valve mechanisms (Duncan & Byrne, 2000; Lehmann, 2001), but must remain open to sustain increased metabolic activity of the wing muscles (e.g. see studies on *Geotrupes* sp., *Onthophagus fracticornis*, *Sysiphus schaefferi*, Miller, 1966). Respiratory water can be lost when spiracles are open, and consequently a larger transpiration surface area may lead to a larger amount of water loss. Smaller relative size of wings may also influence desiccation resistance and tolerance. The wing surface area can be very large compared to the rest of the body, making the wing a potential weak point for water loss through cuticle transpiration. Wings are not just “dead” cuticle, but their veins host living tissues that need supplies of oxygen, water, nutrients, and other substances. Moreover, the wing cuticle must receive a constant supply of water to maintain its elasticity and toughness, and therefore its proper functioning (Pass, 2018). Much evidence has suggested the important role of aptery in reducing water loss in beetles (Scholtz, 1981; Chown et al., 1998; Duncan, 2005), but the wing size of insects is often not reported in water balance studies.

4.2. Thermal tolerance differs among species

Among the tested species, *E. fulvus* and *O. taurus* showed wide ranges in their upper thermal limits. The former reached CT₁₀₀ at 48°C while the latter reached CT₁₀₀ at 45°C. Also *O. fracticornis* reached CT₁₀₀ at 45°C, but the survival curves showed higher mortality of individuals at lower temperatures compared to *O. taurus*. The least tolerant species to high temperatures was *G. stercorarius* (CT₁₀₀=41°C). Our results are in accord with the chorological categories of the four species: *E. fulvus* is classified as a turano-europeo-mediterranean chorotype, *O. taurus* as a

centralasiatic-europeo-mediterranean chorotype, *O. fracticornis* as a turano-european chorotype, while *G. stercorarius* is characteristic of colder climates, and it classified as a euro-siberian chorotype (Carpaneto et al., 2000). *E. fulvus*, *O. taurus*, and *O. fracticornis* belong to the Scarabaeidae which have a Gondwanian origin, and they are more adapted to tropical and hot-temperate conditions (Gallego et al., 2018).

Our data on differences in thermal tolerance of the four species are in accord with the findings of Gallego et al. (2018) who classified *G. stercorarius* as a non-thermoregulator species with an upper critical temperature of $41.7 \pm 1.8^\circ\text{C}$. *G. stercorarius* belongs to the Geotrupidae family, whose origins date back to the Cretaceous period (Krell, 2007; Cunha et al., 2011), and is mainly distributed across the Holarctic regions. We argue that *G. stercorarius* is a non-thermoregulator species lacking physiological adaptations to heat stress since its main physiological requirement is to maintain and generate endogenous heat rather than to eliminate it (Gallego et al., 2018). Differences between species may also be linked to body size, *G. stercorarius* being larger than the other three species. Large endothermic dung beetles may have an advantage in the exploitation of cooler environmental conditions, but heat generation in these insects may represent an obstacle for colonization of those areas with high summer temperatures.

4.3. Evidence of trade-offs between thermal tolerance and water balance strategies

In considering previous results on thermal tolerance and water balance regulation, it should be highlighted that traits are not isolated entities, but features of a single individual. This means that values of traits of the same individual are necessarily interrelated, because they are part of the organism's integrated phenotype (Renault et al., 2005; Hanisch et al., 2020). We found that the species with highest values of upper thermal tolerance and consequently higher survival rates (i.e. *E. fulvus*) was characterized by low desiccation resistance and high desiccation tolerance. Our results suggest that morphological or physiological mechanisms controlling thermal tolerance have a trade-off effect on the length of time that dry conditions may be tolerated (desiccation resistance), while they seem to act synergistically with those mechanisms controlling the proportion of the initial water content that is lost at the time of death (desiccation tolerance). As suggested by Bujan et al. (2016), a possible explanation for the trade-off between thermal tolerance and desiccation resistance lies in the cuticular lipids that coat the exoskeleton and inhibit water loss. Warm temperatures increase cuticular permeability that enhances evaporative water loss (low desiccation

resistance), but favours passive evaporative cooling (high thermal tolerance). Individuals that can tolerate a high amount of water loss (high desiccation tolerance) are those characterized by high thermal tolerance.

Trait-specific constraints may lead to trade-off effects or synergies in the regulation of body temperature and water balance. We found that a large beetle, *G. stercorarius*, was less resistant and tolerant to desiccation than predicted by surface area to volume ratio because of potential trade-offs between thermal tolerance and desiccation resistance (Baudier et al. 2015; Kaspari et al. 2015; Bujan et al., 2016). The advantageous effect of a large body size in dry conditions may be counterbalanced by large spiracles that may increase heat loss, but similarly increase respiratory water loss, especially during flight when the spiracles must remain open to sustain increased metabolic activity of the wing muscles (Lehmann, 2001). On the contrary, small beetles such as *Onthophagus* sp. might be more resistant and tolerant to desiccation than predicted by surface area to volume ratio because small spiracle size increases desiccation resistance and tolerance, but at the same time might reduce heat dispersion when temperatures are high. Insect mortality at high temperatures is closely associated with rapid water loss leading to desiccation (Weldon et al., 2016).

We have shown that thermal tolerance and water balance strategies provide a framework to investigate functional adaptations to joint environmental variation in temperature and water availability, with potential physiological and/or morphological conflicts between thermoregulation and water balance. In this regard, our results seem to suggest that, in a context of climate change, species that are less resistant to desiccation, but more tolerant to water loss, may be the most tolerant to increasing temperatures. The variety of mechanisms that can generate desiccation resistance and tolerance, and that can interact with thermal tolerance, including fluidity of epicuticular lipids, deserves further study as a potential key functional trait in tiny ectotherms.

4.4. Ecological implications

Climate warming has been proposed as the main cause of changes in community structure and turnover of dung beetle species (Dortel et al., 2013; Menéndez et al., 2014; Birkett et al., 2018). These changes will be highly dependent on thermal tolerances and water balance strategies of dung beetles, with species or populations with high maximum thermal tolerance most likely to persist under, or even benefit from, climate change (Deutsch et al. 2008; Khaliq et al. 2014). Dortel et al. (2013) forecast a significant impoverishment of dung beetle richness in south-western Europe with

a potential enrichment in the northern regions due to a northward shift of thermophilous species. These changes may have important consequences for the ecosystem functions and services that dung beetles provide. A potential decline in tunneler species is especially worrying considering their pivotal role in bio-geochemical cycles, including dung decomposition, availability of plant-growth-limiting nutrients, and greenhouse gas emissions from dung (Slade et al., 2007, Nervo et al., 2017, Piccini et al., 2017b).

Our results suggest that higher sensitivity of large species such as *G. stercorarius* to warm temperatures and arid conditions may be relevant in a context of changing ecosystem functions under warming conditions. Body size is a crucial trait that influences the provisioning of ecosystem services such as dung removal, soil nutrient cycling, and vegetation growth (e.g. Nervo et al., 2014; Nervo et al., 2017; Piccini et al., 2017a; Piccini et al., 2017b, de Castro-Arrazola et al., 2020). The replacement of species sensitive to warm temperatures (i.e. *G. stercorarius*) by more resistant species (i.e. *E. fulvus* or *O. taurus*) under a warming climate may influence the provisioning of ecosystem services, since large sized species have a disproportionate effect on ecosystem functioning. Furthermore, some studies have shown that warming temperature and lower humidity decreased brood production and dung burial by tunnelers and rollers (e.g. *O. taurus* and *Sisyphus rubrus*), suggesting the potential effect of climate change on the provisioning of ecosystem services (Holley & Andrew, 2019; Holley & Andrew, 2020). However, a recent study did not find variations in the behavioural plasticity of *O. taurus* in response to warming (Mamantov et al., 2021).

In conclusion, our data suggest temperature and water availability may have interactive effects on dung beetle responses. Environmental changes are multidimensional and responses may be both non-linear and non-additive: a trait that responds well under one environmental stressor, such as increased temperature, may not fare well when another stressor, such as increased aridity, is acting simultaneously (Bubliy et al., 2012).

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APPENDIX

Table A1. Average size values of body mass, abdominal spiracles, mesothoracic spiracles, and wings for the four species used in the experiment. These values were measured on a sample of 87 individuals that were tested for desiccation resistance and tolerance.

Species	Body mass (mg)	Total length of abdominal spiracles (mm)	Area of mesothoracic spiracles (mm ²)	Length of wing (mm)
<i>E. fulvus</i>	0.020±0.005	0.618±0.087	0.190±0.027	3.270±0.178
<i>G. stercorarius</i>	0.286±0.153	2.201±0.266	0.567±0.112	9.246±0.862

<i>O. fracticornis</i>	0.023±0.011	0.545±0.138	0.118±0.036	3.040±0.290
<i>O. taurus</i>	0.024±0.007	0.665±0.081	0.199±0.040	3.220±0.263

Table A2. Differences among species in fractional water content (initial water content/body mass). The reference level for species is *E. fulvus*.

Fractional Water Content (~ species)			
AIC= 278 (sample size = 76)			
	Estimate	t value	p
Intercept	0.95±0.33	2.88	**
<i>G. stercorarius</i>	-0.13±0.38	-0.34	NS
<i>O. fracticornis</i>	0.38±0.38	0.99	NS
<i>O. taurus</i>	-0.10±0.41	-0.25	NS

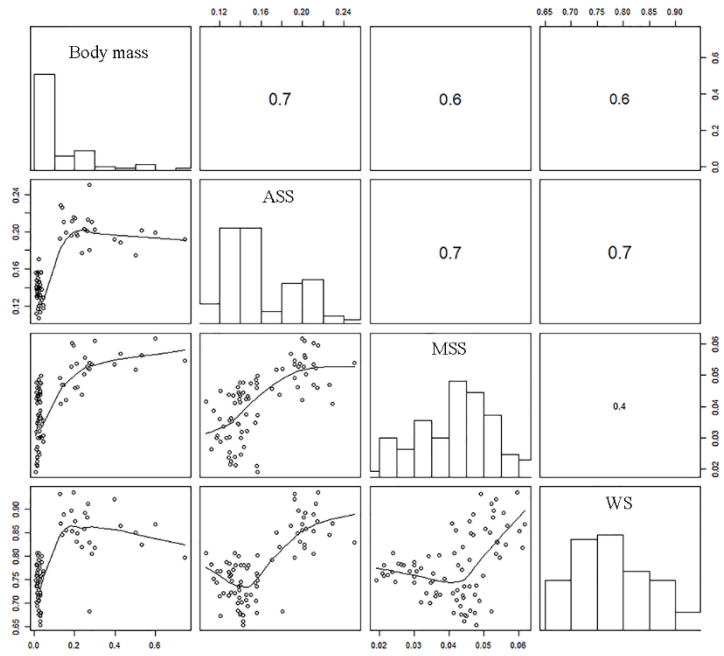
Table A3. Differences among species in body mass and relative size of abdominal and mesothoracic spiracles and wings. The reference level for species is *E. fulvus*.

Body mass (~ species)			
AIC= -388 (sample size = 76)			
Variance explained= 0.90			
Intercept	-3.89±0.13	-30.93	***
<i>G. stercorarius</i>	2.65±0.15	17.41	***
<i>O. fracticornis</i>	0.18±0.15	1.20	NS

<i>O. taurus</i>	0.15±0.16	0.89	NS
Species comparisons			
<i>G. stercorarius</i> - <i>O. fracticornis</i>	2.46±0.12	20.18	**
<i>G. stercorarius</i> - <i>O. taurus</i>	2.50±0.14	18.40	**
<i>O. fracticornis</i> - <i>O. taurus</i>	0.04±0.14	0.27	NS
Abdominal spiracle size (~ species)			
AIC= -445 (sample size = 76)			
Variance explained= 0.83			
	Estimate	Z value	p
Intercept	0.15±0.00	35.39	***
<i>G. stercorarius</i>	0.05±0.00	10.77	***
<i>O. fracticornis</i>	-0.02±0.00	-3.56	***
<i>O. taurus</i>	-0.01±0.00	-1.40	NS
Species comparisons			
<i>G. stercorarius</i> - <i>O. fracticornis</i>	0.43±0.02	17.59	**
<i>G. stercorarius</i> - <i>O. taurus</i>	0.35±0.03	13.04	**
<i>O. fracticornis</i> - <i>O. taurus</i>	0.07±0.03	-2.74	**
Mesothoracic spiracle size (~ species)			
AIC= -593 (sample size = 76)			
Variance explained= 0.73			
Intercept	-3.12±0.05	-58.41	***
<i>G. stercorarius</i>	0.17±0.06	2.79	**
<i>O. fracticornis</i>	-0.40±0.06	-6.46	***
<i>O. taurus</i>	-0.03±0.07	-0.54	NS
Species comparisons			
<i>G. stercorarius</i> - <i>O. fracticornis</i>	0.02±0.00	14.25	***
<i>G. stercorarius</i> - <i>O. taurus</i>	0.01±0.00	5.51	***

<i>O. fracticornis</i> - <i>O. taurus</i>	-0.01±0.00	-7.22	***
Wing size (~ species)			
AIC= -297 (sample size = 76)			
Variance explained= 0.74			
Intercept	-0.28±0.02	-16.36	***
<i>G. stercorarius</i>	0.07±0.02	3.22	**
<i>O. fracticornis</i>	-0.02±0.02	-0.86	NS
<i>O. taurus</i>	-0.09±0.02	-4.04	***
Species comparisons			
<i>G. stercorarius</i> - <i>O. fracticornis</i>	0.09±0.10	9.27	***
<i>G. stercorarius</i> - <i>O. taurus</i>	0.16±0.11	14.35	***
<i>O. fracticornis</i> - <i>O. taurus</i>	-0.07±0.11	5.97	***

Figure A1. Pairplot of predictor variables: body mass, relative size of abdominal spiracles, mesothoracic spiracle and wing. The upper panel contains estimated pair-wise correlations, and the font size is proportional to the absolute value of the estimated correlation coefficient. The diagonal panel contains histograms and the lower panel scatterplots with a LOESS (locally weighted smoothing) smoother added to aid visual interpretation. The degree of correlation is high (>0.5) for body mass and the other three morphological traits, for ASS and MSS, and for ASS and WS.



Vitae

Beatrice Nervo is an ecologist with expertise in the functional ecology of dung beetles. She works on response traits to environmental factors and effect traits that affect the provisioning of ecosystem services.



Angela Roggero is an entomologist whose research is mainly focused on dung and ground beetles. Her interests include systematics, evolutionary biology, phylogeny and biogeography. Currently she is studying form and function of morphological traits applying geometric morphometric methods.



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Claudia Palestrini is an entomologist with competence in dung beetle morphology, ecology and systematics.



Antonio Rolando is an animal ecologist devoted to the study of birds (human impact on communities and populations) and dung beetles (ecological functions and services).

